Reference: *Biol. Bull.*, **142**:407–416. (June, 1972)

EVAPORATIVE WATER LOSS BY TARDIGRADES UNDER CONTROLLED RELATIVE HUMIDITIES

JOHN H. CROWE

Department of Zoology, University of California, Davis, California 95616

Certain soil-dwelling animals such as rotifers, tardigrades, and nematodes, possess the unique ability, under drought conditions, to be divorced from virtually all their body water. Yet they are not killed. When they come in contact with water the animals swell and resume active life, often within minutes. This phenomenon, which has fascinated biologists since its discovery by Leeuwenhoek, is called cryptobiosis (see Keilin, 1959; Crowe, 1971; Crowe and Cooper, 1971 for recent reviews).

Tardigrades which survive desiccation must be allowed to dry very slowly ("très lentement," according to Broca, 1860, page 1) but exactly what "very slowly" means in quantitative terms or of what significance the slow drying might be to the animal has been only a source of conjecture.

Davis (1873) believed that slow drying of rotifers enabled the animals to secrete a gelatinous sheath which protected them from desiccation. Jacobs (1909) and Hickernell (1917) disproved this notion by chemical tests for water and direct histological examination, which revealed no such sheath. Others, notably Pouchet (1859a, 1859b), observed that tardigrades or rotifers dried on a clean slide were killed, while those dried in a little sand survived. They believed that sufficient water remained between the sand grains to maintain the animals in a state of partial hydration. Doyère (1842) on the other hand, insisted that drying on a clean slide was not necessarily injurious. Close examination of Doyère's techniques shows, however, that he dried his animals slowly, in a humid atmosphere, while Pouchet took no such precautions. Clearly, the humidity of the surrounding air, and presumably the consequent rate of evaporative water loss, are important to the tardigrade that is to become cryptobiotic.

The focus of this investigation is on the rate of evaporative water loss from tardigrades kept under controlled relative humidities. The aim of the study is to provide a basis for investigations on the mechanism of the induction of cryptobiosis.

Experiments and Observations

Evaporative water loss

The rate of evaporative water loss from tardigrades was investigated by recording weight loss from animals kept at known relative humidities. Specimens of *Macrobiotus areolatus* Murray, 1907, which had been starved for 24 hours immediately before the experiment, were washed in distilled water, picked up individually with an Irwin loop, and blotted with filter paper to remove adhering water. The blotted animals were transferred with an Irwin loop (which was silicone-

coated to prevent removal of water from the test droplet) to a precisely measured 5 or 10 μl drop of distilled water on a 5 mm weighing pan. The weighing pan was suspended from the pivot arm of a Cahn RG electrobalance calibrated to ± 0.54 μg. The balance was coupled to a Sargent chart recorder. Relative humidity of the air surrounding the pan was controlled by sulfuric acid solutions of known density (and therefore of known vapor pressure; *cf.* Edney, 1966). The flask containing the sulfuric acid solution was provided with a magnetic stirring bar to insure fast equilibration between the solution and air. The entire apparatus was housed in a constant temperature chamber at 25° C.

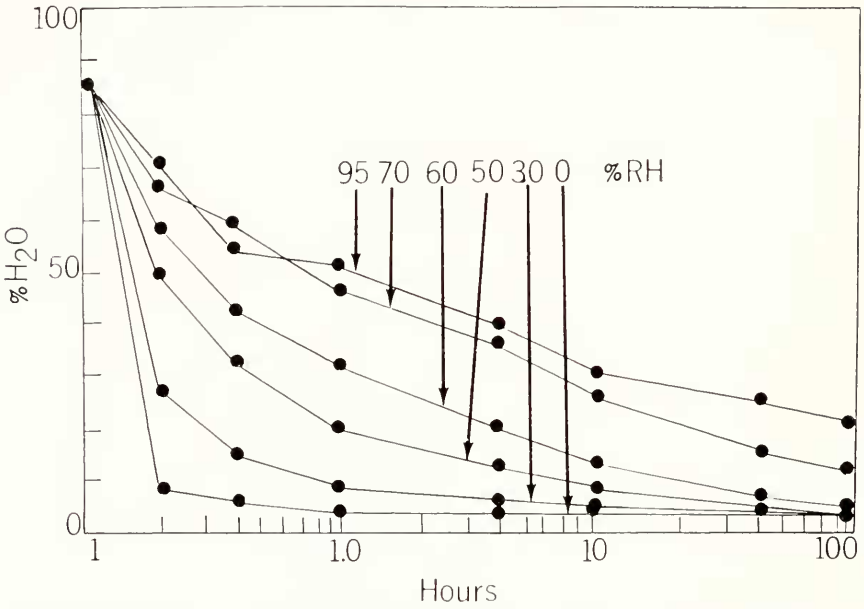

FIGURE 1. Water contents of tardigrades kept at various relative humidities.

The results of these experiments are presented in Figures 1 and 2. Calculation of the per cent water contents was based on the assumption that animals held at 180° C for 3 hours contained no water. Calculations were made according to the equation: $\%\ H_2O\ \text{content} = \dfrac{W_t - W_d}{W_t} \times 100$,

where W_t = weight at time t and W_d = dry weight. The weight of an average, fully hydrated animal was about 98 μg, of which about 12 μg was dry matter and 86 μg was water. All determinations were carried out on groups of about 25 animals.

Over the range of about 70–95% relative humidity (RH) different groups of animals which were active at the beginning of the experiment showed similar rates of evaporative water loss. During the first hour under these conditions active animals were reduced from their original water contents of 80–90% to about

50–60% (Figs. 1 and 2). Active animals were subsequently reduced to an equilibrium water content of 10–25% over a period of about 100 hours.

At relative humidities below 60%, the rate of evaporative water loss by animals which were active at the beginning of the experiment was much greater than at higher humidities (Fig. 1). At 0% RH, for example, the animals were reduced to 2–3% water content in about 15 minutes. Active animals dried under these conditions were killed. Even though such animals could not survive direct exposure to dry air, those which were reduced previously to about 20% water content at 80% RH did survive exposure to dry air. When such animals were placed in dry air, their water content was reduced to 2–3% without killing them.

The differences in the rates of water loss from animals which were active at the beginning of the experiment and animals which were dead or anesthetized animals were highly significant ($P = 0.01$). Animals which were active at the beginning of the experiment retained a significant proportion of their body water for extended periods, while animals which were killed with 2% formalin or 0.1 M KCN or anesthetized with CO_2 were reduced to equilibrium water content within one hour. Active animals lost water at a rate approximately 0.3 times that of anesthetized animals during the first hour under dehydrating conditions. Anesthetized animals not dried regained activity within a few minutes when they were returned to oxygenated water, but the dried animals were killed.

Permeability coefficients (K) were calculated for four arbitrarily selected time periods (Figure 2), according to the equation of Lucké and McCutcheon (1932):

$$K = \frac{dV}{dt(P_1 - P_2)A},$$

where dV = volume of water lost (liquid phase),
dt = time change,
$P_1 - P_2$ = net water potential, and
A = surface area.

A 500 μ long animal with surface area of 2×10^{-4} cm^2 was assumed. Hemolymph osmotic pressure (OP) needed to make these calculations was measured by the indirect freezing point method of Gross (1954). OP showed a range of 2.20–2.87 osmoles/l, with a mean of 2.48 osmoles/l.

During the first stages of dehydration the K's were similar over the entire humidity range, but the K for anesthetized animals was consistently higher than that for animals active at the beginning of the experiment. The K for anesthetized animals dried at 80% RH was nearly 3 times that of active animals dried at the same humidity. In the latter stages of dehydration, the K of animals dried at RH > 70% decreased as much as 100-fold (Fig. 2).

Morphology and cuticular permeability

Active animals (Fig. 3) contract when dried at high humidities and form so-called "tuns" (Fig. 4). Anesthetized animals, on the other hand, simply flatten (Fig. 6) or crumple (Fig. 5).

Crowe, Newell and Thomson (1971a) reported that the cuticular folds (Figure 4), which are flexion points in the cuticle, are about 1/20 the thickness of the rest of the cuticle. This observation suggests that these thin areas may be more highly permeable than the rest of the cuticle. When the animal contracts into

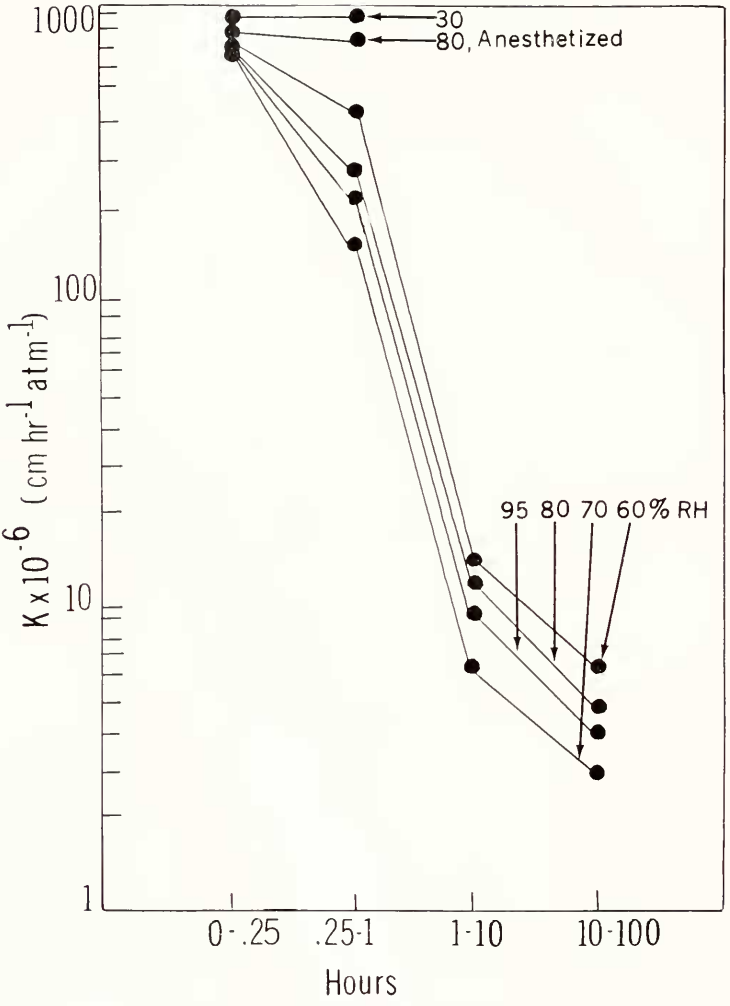

FIGURE 2. Permeability coefficients of tardigrades dried at various relative humidities. For explanation see text.

a tun (Fig. 4), the thin cuticle areas are drawn into the body wall and removed from contact with the air. If such areas were indeed more permeable than the rest of the cuticle, formation of the tun could then, in part, account for reduced evaporative water loss by tuns compared with animals which were not allowed to form tuns.

Relative permeabilities of the thick and thin areas of the cuticle were studied through the use of dyes. Active animals were immersed in 0.5% crystal violet, periodically transferred to distilled water, and examined with the light microscope. The dye penetrated the thin portions of the cuticle within a few seconds,

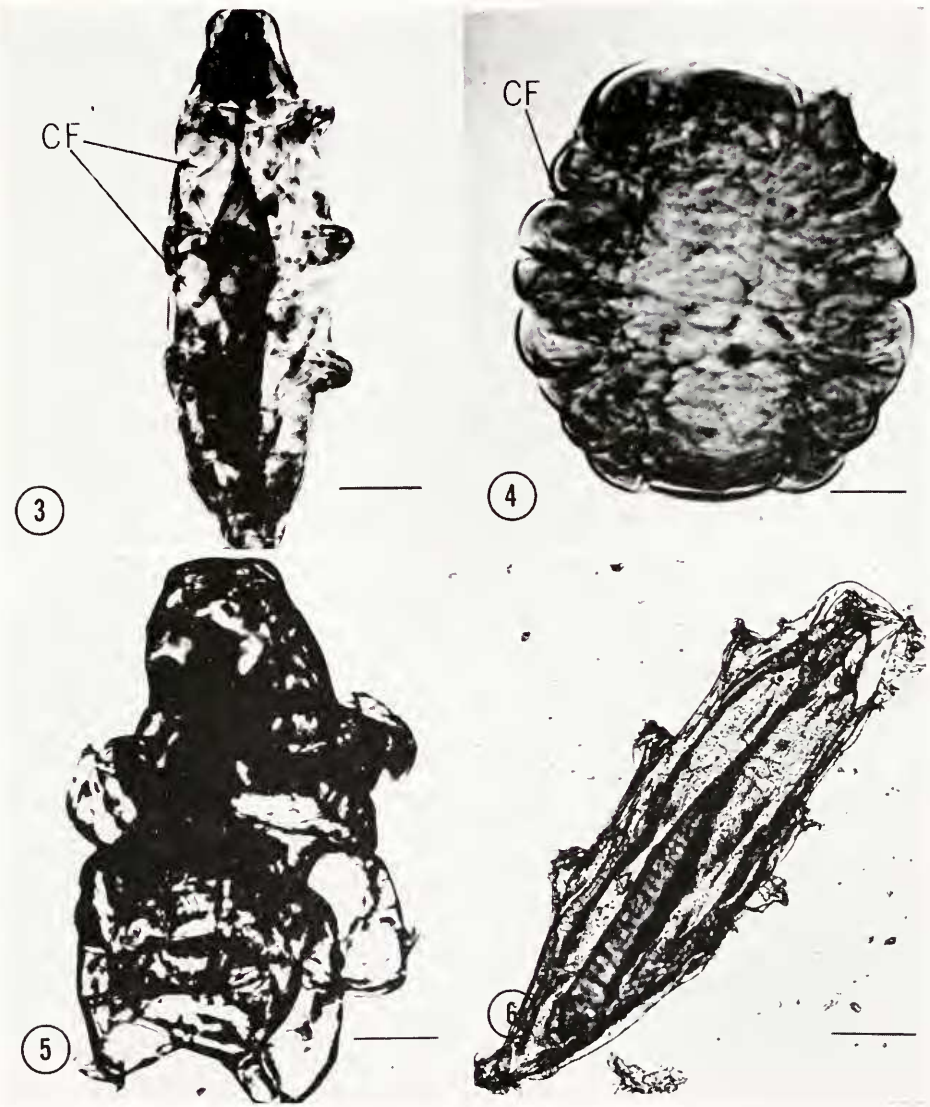

FIGURE 3. Photomicrograph of an active tardigrade; CF = cuticular folds; marker = 50 μ.

FIGURE 4. A tun of *M. arcolatus*, obtained by drying an active animal at high humidity; CF = cuticular folds; marker = 50 μ.

FIGURE 5. A specimen of *M. arcolatus* that was dried at low humidity; marker = 50 μ.

FIGURE 6. A specimen of *M. arcolatus* that was anesthetized before it was dried; marker = 50 μ.

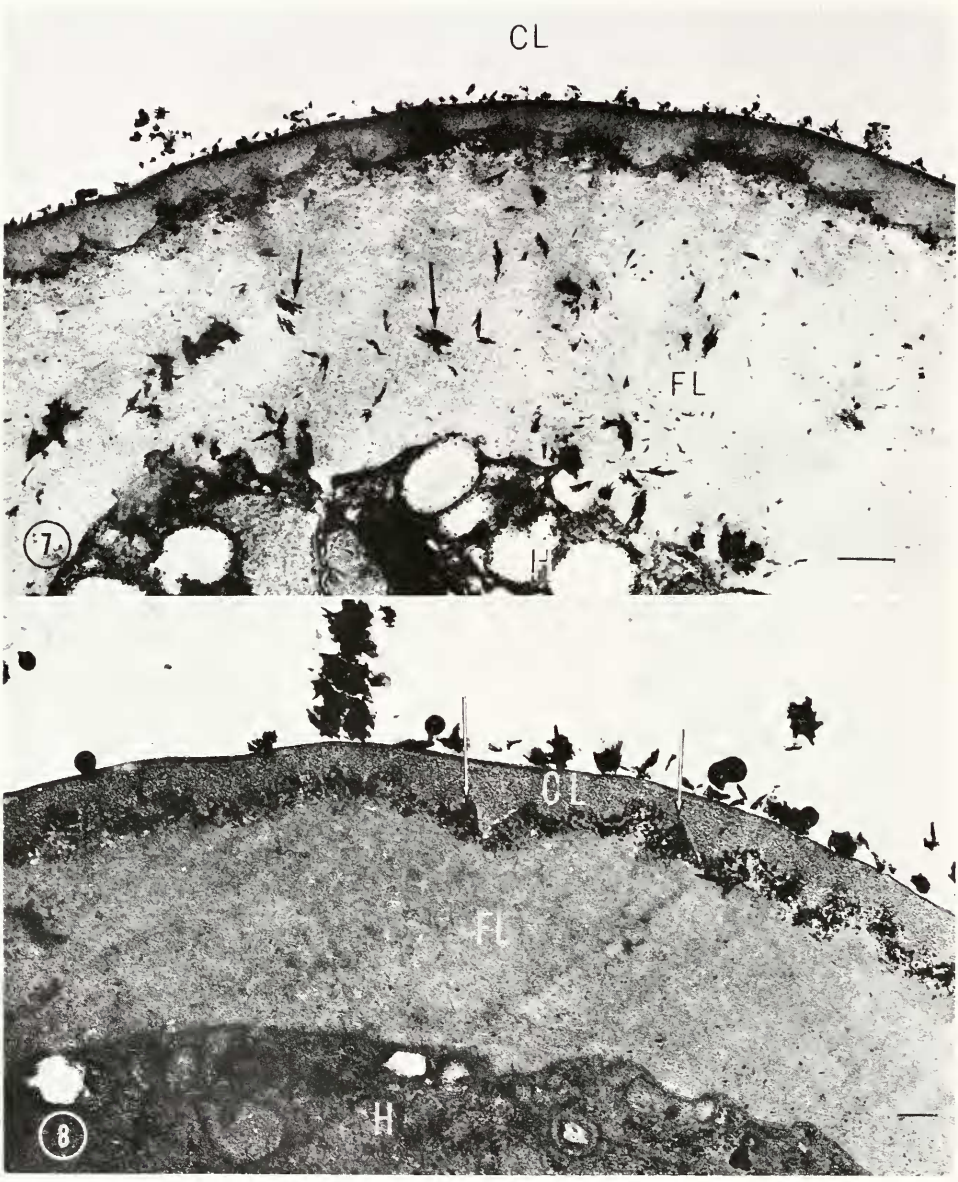

FIGURE 7. Electron micrograph showing the penetration of the cuticle of *M. arcolatus* in the active state by lead ions. Note the distribution of lead (arrows) throughout the cuticle and hypodermis; CL = cortical layer; FL = fibrous layer; H = hypodermis; marker = 1 μ.

FIGURE 8. Electron micrograph showing the penetration of the cuticle of *M. arcolatus* in the cryptobiotic state by lead ions. Note the accumulation of lead (arrows) at the outer boundary of the fibrous layer; marker = 1 μ.

but it penetrated the thicker portions extremely slowly. Crystals were seen in the hypodermis underlying the thick cuticle only after prolonged exposures of several hours. Alternatively, the animals were vitally stained for 24 hours in 0.1% neutral red, a stain which changes to yellow at alkaline pH. Animals so stained were placed in water at pH 8.5. The color change was seen most intensely at the mouth and anus and along the cuticular folds. It appears, then, that the cuticular folds are areas of high permeability as their morphology suggests.

Sequestration of the high permeability areas of the cuticle by tuns can account for the differences between rates of evaporative water loss by active and anesthetized animals, but it clearly cannot account for the 100-fold decrease in PC seen in the terminal stages of dehydration. This diminution in rate of water loss suggests that the entire cuticle decreases in permeability as it dries. This possibility was investigated by comparing rates of water entry into active tradigrades and tuns. The animals were stained with neutral red as previously described. Active animals placed in water at pH 8.5 showed a color reaction at the mouth, anus, and cuticular folds most strongly, but also showed a weak reaction along the thick cuticle, indicating that at least some water penetrates through this cuticle. Tuns placed in water at pH 8.5, on the other hand, showed a strong reaction at the mouth and anus, but no reaction in other portions of the cuticle for several seconds. These results, which suggest that the cuticle does indeed decrease in permeability when dried, were confirmed in the following way. Active animals and tuns were placed in 2% aqueous $PbNO_3$ for 10 sec and immediately transferred to 4% paraformaldehyde in 0.1 m phosphate buffer, the phosphate ions trapping Pb^{++} as $PbPO_4$. The animals were then processed for electron microscopy as previously described (Crowe *et al.*, 1971a). The results of this experiment show that lead penetrates the cuticle readily; $PbPO_4$ crystals were seen scattered through the cortical and fibrous layers of the cuticle of active animals (Fig. 7). In tuns, however, penetration is much reduced; $PbPO_4$ crystals were seen only in the cortical layer, never in the fibrous layer (Fig. 8).

Discussion

These studies have provided a more precise analysis of the effects of relative humidity on survival and desiccation rate of tardigrades in air than was previously possible. The highest number of survivals was obtained when the animals were dried at relative humidities greater than 70%. This is in some way related to the fact that at high humidities the tardigrades retained significant proportions of their body water for extended periods of time—up to about 100 hours (Figs. 1 and 2). At lower relative humidities they lost water at a much greater rate, reaching equilibrium within 1 hour (Fig. 1). At high humidities the animals contract into a tun (Fig. 4), while anesthetized animals or animals dried at lower humidities become irregularly crumpled (Fig. 5) or simply flattened (Fig. 6). The difference in the rate of water loss by tuns and non-tuns is probably due to the fact that in tuns, high permeability areas of the cuticle are removed from contact with the air.

The rate of evaporative water loss continues to fall for some time, even after the animals have contracted into tuns; during the first hour at 80% RH tuns lose water at a rate of about 34 mgm cm^{-2} hr^{-1}, 9 mgm cm^{-2} hr^{-1} during the 1–10

hour period, and 0.7 mgm cm^{-2} hr^{-1} during the 10–100 hour period (calculated from Fig. 1 and 2). These rates are similar to those shown by aquatic insects in air. Holdgate (1956) found that evaporative water loss from such insects was 0.3–11.5 mgm cm^{-2} hr^{-1}. The decrease in rate of evaporative water loss appears, from the evidence presented above, to be due to a decrease with desiccation of the permeability of the cuticle to water. Such decreases are not unknown. For example, Edney (1951) Auzon (1953), and Bursell (1955) found that when isopods are exposed to dehydrating conditions, the rate of evaporative water loss falls for some time before it reaches steady state. A similar phenomenon has been reported for certain aquatic insects by Bursell (1955) and Holdgate (1956). Conversely, most terrestrial insects achieve constant rates of evaporative water loss rather quickly (Beament, 1958, 1961).

It is significant that the arthropods that exhibit decreases in cuticular permeability under dehydrating conditions lack extra-cuticular lipids, but possess endocuticles that are rich in lipo-proteins (see Edney, 1957 for references). The cuticle of *M. arcolatus* also lacks extra-cuticular lipids, but the fibrous layer of the cuticle (which appears to be the major permeability barrier) is rich in lipoproteins (Crowe *et al.*, 1971). Bursell (1955) suggested that dehydration of the cuticles of isopods brings epicuticular lipids in close proximity to each other, which results in a permeability decrease.

If the cuticle is really important to the tardigrade in controlling its rate of desiccation, an obvious difficulty is the molt. Crowe, Newell and Thomson (1971b) have shown, however, that in *M. arcolatus* the old cuticle is maintained intact until the new cuticle is completed. It appears that the old cuticle retains its function until the new one is completed.

It seems now that slow evaporative water loss is necessary for the survival of desiccation by tardigrades. Similar observations have been made by Ellenby (1968). He found that nematodes undergoing desiccation congregate into groups, forming a "nematode wool." Those individuals on the inside of the group dry more slowly and survive desiccation better than those on the outside. Ellenby also presented some evidence that the cuticle of nematodes undergoing desiccation decreases in permeability.

The most interesting questions yet to be answered center around the necessity for slow evaporative water loss by animals entering a cryptobiotic state. That water loss must be slow implies that the animals must prepare in some way for extensive desiccation. I have presented hypotheses elsewhere concerning the nature of the preparative process (Crowe, 1971; Crowe and Cooper, 1971).

I gratefully acknowledge the advice and encouragement of E. B. Edney, I. M. Newell and W. W. Thomson during these studies. Supported in part by grants ES00084 from the National Institute of Environmental Health Sciences and D-686 from the University of California.

Summary

The effects of relative humidity on the survival of desiccation by the tardigrade, *Macrobiotus arcolatus*, were investigated. The most survivals were obtained when

the animals were dried at relative humidities greater than 70% at 20° C. At these high humidities the animals form tuns, while at lower humidities they become flattened or crumpled. Anesthetized animals do not form tuns at any humidity.

The rate of evaporative water loss from tuns in air was investigated by recording weight loss from animals kept at known relative humidities. Tuns formed by active animals lose water during the early stages of dehydration at a rate approximately 0.3 times the rate of anesthetized animals. Anesthetized animals equilibrate with the surrounding air within one hour, while tuns require more than 100 hours to equilibrate. At the end of 100 hours, the water content of tuns at 80% RH is 10–25%. During dehydration the permeability coefficient of tuns decreases a hundredfold (from $2.0–5.0 \times 10^{-4}$ cm hr^{-1} atm^{-1} to $1.6–6.0 \times 10^{-6}$ cm hr^{-1} atm^{-1}). The tardigrades can be reduced to 2–3% water content in dry air without killing them, if they are first dehydrated to about 20% water content at 70–95% RH.

The cuticle was investigated as the most likely site of control of evaporative water loss. Studies with dye and heavy metal tracers demonstrated that during tun formation high permeability areas of the cuticle are removed from contact with the air. After extensive dehydration the entire cuticle becomes less permeable to water, possibly due to a lipid phase change.

LITERATURE CITED

Auzou, M. L., 1953. Recherches biologique et physiologiques sur deux onisciens, *Porcellio scaber* et *Oniscus ascellus*. *Ann. Sci. Nat.*, **15**: 71–98.

Beament, J. W. L., 1958. The effect of temperature on the waterproofing mechanism of an insect. *J. Exp. Biol.*, **35**: 494–519.

Beament, J. W. L., 1961. The water relations of the insect cuticle. *Biol. Rev.*, **36**: 281–320.

Broca, P., 1869. Rapport sur la question soumise à la Société de Biologie au sujet de la reviviscences des animaux desséchés. *Mem. Soc. Biol., Paris*, **2**: 1–140.

Bursell, E., 1955. The transpiration of terrestrial isopods. *J. Exp. Biol.*, **32**: 238–255.

Crowe, J. H., 1971. Anhydrobiosis: an unsolved problem. *Amer. Natur.*, **105**: 563–574.

Crowe, J. H., and A. F. Cooper, Jr., 1971. Cryptobiosis. *Sci. Amer.*, **225**: 30–36.

Crowe, J. H., I. M. Newell and W. W. Thomson, 1971a. Fine structure and chemical composition of the cuticle of the tardigrade, *Macrobiotus arcolatus* Murray. *J. Microscopic*, **11**: 10–23.

Crowe, J. H., I. M. Newell and W. W. Thomson, 1971b. Cuticle formation in the tardigrade, *Macrobiotus arcolatus* Murray. *J. Microscopic*, **11**: 24–33.

Davis, H., 1873. A new Callidina: with the results of experiments on the desiccation of rotifers. *J. Roy. Microscop. Soc.*, **9**: 201–209.

Doyère, P. L. N., 1842. Mémoirs sur les Tartigrades. Sur la faculté que possédent les Tardigrades, les Rotifères, les Anguillules des toits et quelques autres animalcules, de revenir à la vie après avoid été complétement desséchés. *Ann. Sci. Nat.*, **18**: 5–35.

Edney, E. B., 1951. The evaporation of water from woodlice and the millipede *Glomeris*. *J. Exp. Biol.*, **28**: 91–115.

Edney, E. B., 1957. *The Water Relations of Terrestrial Arthropods*. Cambridge Press, Cambridge, 109 pp.

Edney, E. B., 1966. Absorption of water vapour from unsaturated air by *Arenivaga* sp. (Polyphagidae, Dictyoptera). *Comp. Biochem. Physiol.*, **19**: 387–408.

Ellenby, C., 1968. Desiccation survival in plant parasitic nematodes, *Heterodera rostochiensis* Wollenweber and *Ditylenchus dipsaci* (Kuhn) Filipjev. *Proc. Roy. Soc London Series B*, **169**: 202–213.

Gross, W. J., 1954. Osmotic responses in the sipunculid *Dendrostomum zostericolum*. *J. Exp. Biol.*, **31**: 402–423.

Hickernell, L. M., 1917. A study of desiccation in the rotifer, *Philodina roscola*, with special reference to cytological changes accompanying desiccation. *Biol. Bull.*, **32**: 343–397.

Holdgate, M. W., 1956. Transpiration through the cuticles of some aquatic insects. *J. Exp. Biol.*, **33**: 107–118.

Jacobs, M. H., 1909. The effects of desiccation on the rotifera *Philodina roseola*. *J. Exp. Zool.*, **6**: 207–263.

Keilin, D., 1959. The problem of anabiosis or latent life: history and current concept. *Proc. Roy. Soc. London Series B*, **150**: 149–191.

Lucké, B., and M. McCutcheon, 1932. The living cell as an osmotic system and its permeability to water. *Physiol. Rev.*, **12**: 68–139.

Pouchet, F., 1859a. Nouvelles expériences sur les animaux pseudoressuscitants. *C. R. Acad. Sci., Paris*, **49**: 492.

Pouchet, F., 1859b. Expériences sur la resistance vitale des animalcules pseudoressuscitants. *C. R. Acad. Sci., Paris*, **49**: 886.